Genetic Diversity of *cry* Gene Sequences of *Bacillus thuringiensis* Strains Analyzed by Denaturing Gradient Gel Electrophoresis

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Abstract PCR has been widely used to identify *cry*-type genes, to determine their distribution, to detect new such genes and to predict insecticidal activities. We describe here a molecular approach to analyze the genetic diversity of *B. thuringiensis cry*-like genes based on denaturing gradient gel electrophoresis (DGGE). This analysis demonstrated that different *B. thuringiensis* isolates can be distinguished according to its PCR-DGGE profile of *cry*-like genes. Identification of the resolvable DNA fragments was easy to accomplish by DNA sequencing, which was confirmed in this work. Importantly, the strategy allowed the identification of unknown *B. thuringiensis cry*-like sequences present in a single strain that remained cryptic after PCR analysis using degenerate primers. The method developed in this work contributes to the availability of molecular techniques for both *B. thuringiensis* strains and *cry*-like genes identification and discovery.

Introduction

Cry proteins from the Gram-positive bacterium *Bacillus thuringiensis* have been used as a successful biological insecticide and are extensively used worldwide in programs for controlling insect pests [18]. The significant amount of available Cry proteins is the result of a systematic international effort to isolate and characterize new strains of *B. thuringiensis* searching for toxins with novel properties, particularly appropriate for the control of agronomical or medically important pests [15]. To date, a great amount of proteins has been described, however, to find new Cry proteins with novel receptor specificities in pests will be a way to expand the host ranges of the available strains. On the other hand, it will be a useful alternative after the appearance of insect resistance, mainly due to the use of transgenic crops.

DGGE is an electrophoretic method capable of detecting differences between DNA fragments of the same size but with different sequences. This is because the fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profile. Separation is based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants. The melting of DNA fragments proceeds in discrete, "so called", melting domains: stretches of base pairs with an identical melting temperature. While DGGE was originally developed to detect point mutations in DNA sequences, Muyzer et al. [12] expanded its use to study microbial genetic diversity in a marine ecosystem. For this purpose, total bacterial DNA was extracted from natural samples, segments of the 16S or 18S rRNA genes were amplified in the polymerase chain reaction and the individual products were subsequently separated by DGGE. The result was a pattern of bands, for which their number corresponded to the number of predominant members in the microbial communities allowing to compare them in different environments and to monitor changes in the composition of abundant members of a specific community through out the time. In the last years, DGGE of PCR amplified 16S rRNA and 18 rRNA genes fragments has been applied to profile the distribution of microbial populations in a great variety of environments like soil,

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phyllosphere, rhizosphere, freshwater lake, seawater samples, foods and among others samples [4, 5, 7, 10, 19]. Using DGGE many samples taken at different intervals during the study or samples with different treatments can be simultaneously analyzed. This makes the technique a powerful tool for monitoring specific genes behaviour after periods of time or environmental changes [9, 11].

The main goal of this work was to develop a method, based on PCR-DGGE, for the direct analysis of the genetic diversity of a mix of *cry*-related sequences present in a single *B. thuringiensis* strain or, eventually, in an environmental sample. To fulfill this objective, specific primers designed for the *cry* genes identification, adapted to the DGGE technique were used. This procedure allows a direct identification of the presence of different *cry*-related sequences in a single or a mixture of *B. thuringiensis* strains.

Materials and Methods

Bacterial Strains and Growth Conditions

Reference *B. thuringiensis* strains serovar. *thuringiensis* (HD1), *darmstadiensis* (4M1), *tenebrionis* (Btt), *sotto* (4E1), *aizawai* (4J3), *kenyae* (4F1), *galleriae* (4G1), *israelensis* (H14), *morrisoni* (4K1), *wuhanensis* (4T1), *kumamtoensis* (4W1), *fukuokaensis* (4AP1) and *jegathesan* (4CF1) were kindly supplied by D. Zeigler (*Bacillus* Genetic Stock Centre, Columbus, OH, USA). Argentinean native strains were obtained from the FIBA Culture Collection (Fundación para Investigaciones Biológicas Aplicadas, Mar del Plata, Argentina). Cultures of *B. thuringiensis* strains were grown at 28°C in nutrient broth (Difco) with vigorous shaking or on nutrient broth agar. Cells of transformed *Escherichia coli* DH5α were grown at 37°C in Luria-Bertani medium supplemented with 50 μg of ampicillin per ml [17].

DNA Extraction and PCR Conditions

The genomic DNA of *B. thuringiensis* strains used for PCR amplification was isolated as previously described [3, 17]. In a first PCR step, we used a degenerate primer pair (OL1-I/OL5) resulted from modifications of previously described oligonucleotides [2]. In OL1-I and OL3 inosines were substituted by other bases at specific positions (Table 1). The PCR reactions were carried out by using 0.2 μg of DNA template in a mixture (total volume, 25 μl) containing each deoxynucleoside triphosphate at a concentration of 400 μM, each primer at a concentration of 2 μM, and 0.5 U of *Taq* DNA polymerase in the corresponding reaction buffer (Invitrogen). Amplifications were performed with a Mastercycler thermal cycler (Eppendorf) under the following conditions: 2 min of denaturation at 94°C, followed by 35 cycles of denaturation for 45 s at 94°C, annealing for 1 min at 35°C, and extension for 1 min at 72°C. An extra extension step consisting of 3 min at 72°C was added after completion of the 35 cycles. The obtained amplicons were used in a second (semi-nested) PCR for DGGE, in which *cry* degenerate primers OL3-I GC and OL5 were used (Table 1). GC clamp (5′ CGCCC GCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG 3′) was attached to the 5′ end of the forward primer so that it could be used in a DGGE system [13]. For the reaction mix 1 μl of a 1:50 dilution of each PCR product was used as a template, 0.5 μM of each primer, each deoxynucleoside-triphosphate at a concentration of 300 μM and 0.5 U of *Taq* DNA polymerase in the corresponding reaction buffer (Invitrogen) in a reaction mixture (total volume, 25 μl), under the following conditions: initial denaturation

Table 1 Characteristics of general and DGGE primers used in this study

Primer pair	Primer sequence[a]	Position[b]	Product size (bp)[c]
OL1-I (f)	5′ TAICAIYATAYGCACARGCIGCMAAYTTHCAT 3′	510–1788	1,308
OL5 (r)	5′ GGAATAAATTCRATTYTRTCTATATAAA 3′		
OL3-ICG (f)	5′ TATBRIRYDRGIDTYCGITATGCT CGCCCGCCG CGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG 3′	1566–1788	252
OL5 (r)	5′ GGAATAAATTCRATTYTRTCTATATAAA 3′		
OL3-I (f)	5′ TATBRIRYDRGIDTYCGITATGCT 3′	1566–1788	252
OL5 (r)	5′ GGAATAAATTCRATTYTRTCTATATAAA 3′		

f forward primer, *r* reverse primer

[a] The sequences correspond to degenerate primers, and they are indicated according to the degenerate DNA genetic code as follows: B = C, G, or T; D = A, G, or T; H = A, C, or T; M = A or C; R = A or G; Y = T or C; and I = A, C, G, or T

[b] Position starting from the first base of the start codon sequence of the *cry1Aa* gene (accession number M11250) according to sequences obtained from the National Center for Biotechnology Information database

[c] Product sizes calculated from *cry* gene sequence obtained from *cry1Aa* (accession number M11250)

of double-stranded DNA for 5 min at 94°C; 10 (touch-down) cycles consisting of 1 min at 94°C, 1 min at 45°C, and 3 min at 72°C with a decrease in the annealing temperature of 1°C per cycle; 20 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C; and extension for 10 min at 72°C. PCR products were analyzed by electrophoresis in 1% (wt/vol) agarose gels in tris–acetate buffer and ethidium bromide staining [17].

DGGE Optimization

DGGE was performed with the DGGE-2001 system (C.B.S. Scientific Co, USA). PCR products (≈ 120–180 ng) amplified with OL3-I GC/OL5 primers were applied to individual lanes in the gel. Electrophoresis was performed in 0.75-mm-thick 6% polyacrylamide gels (ratio of acrylamide to bis-acrylamide, 37.5:1) submerged in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 7.4) at 60°C in accordance with Diez et al. [4]. To obtain the best separation of different DNA fragments, the gradient and the length of electrophoresis had to be optimized. The following electrophoresis conditions were selected based on the results of different DGGE denaturing-agent gradients and time of travel experiments (Fig. 1a, 1b and 1c): 22 h at 60 V, in a linear 30 to 65% denaturant agent gradient (the 100% denaturant solution contained 7 M urea and 40% [vol/vol] deionized formamide). After electrophoresis, the gels were stained for 40 min with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands) and were photographed under UV light using a Photodyne system apparatus (Photodyne Incorporated, Hartland, WI, USA). After this some bands were cut out of the gels and were used as template in a new PCR reaction with the primers OL3-I and OL5 and the same conditions firstly described, for its cloning, sequencing and identification.

## Cloning, Sequencing, and Analysis of the PCR Fragments

PCR amplified products obtained from the amplification of standard strains with the OL3-I/OL5 primers (Table 1) were ligated into the cloning vector pGEM-T Easy (Promega, Madison, Wis.), which was used for transforming *E. coli* DH5α by standard protocols [17]. DNA sequencing was carried out by Macrogen Services (Korea). BLAST X (version 2.2.6) was used for DNA sequence analyses [1]. The identification of the known Cry sequences was obtained from the nonredundant protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Results and Discussion

Primers and PCR Conditions

In a previous work, we have reported a two-step PCR-based approach which allowed us the amplification of unknown *cry*-related sequences that could be characterized after DNA sequencing. We designed a set of five degenerate primers to be used in PCR amplification in five pair combinations that identify coding sequences for either known or unknown members of diverse Cry proteins family [2]. In this work, we applied a similar strategy using the primer pair OL1/OL5 in a first step and in a second step, the primers pair OL3/OL5. Degenerate primers with

Fig. 1 Parallel DGGE gel with PCR products obtained with the primer pair OL3-I GC and OL5 from a mix of different *B. thuringiensis* strains (4T1, 4G1 and HD1). Denaturant gradients were **a** 40 to 80% **b**, **c** 30 to 65% and electrophoresed at 100 V. Samples were electrophoresed for: **a** and **b**, *1* 18 h, *2* 16 h and *3* 14 h; **c** *1* 14 h, *2* 12 h and *3* 10 h
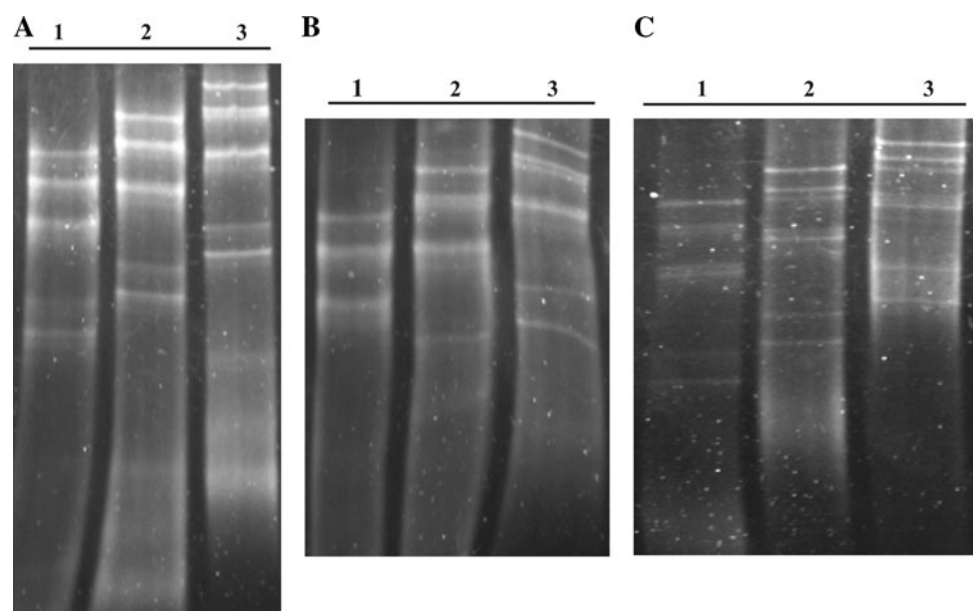
mixed bases have been used in many applications, such as identification of members of gene families. When complete degeneracy is required for a given position, the natural base inosine could be used. Substitution of a mixed-base position with deoxyinosine (dI) was reported to make the amplification more effective [6, 8, 16]. To improve OL primers, some degenerate positions were changed for inosines (Table 1). On the other hand, a GC clamp for DGGE was introduced in primer OL3-I. The primer pair election was according to the amplicon size because the fragments to be resolved by DGGE cannot be longer than 500 bp [14].

Using the primer combination OL1-I/OL5 or OL3-I-CG/OL5 we obtained DNA fragments of 1300 or 200–300 bp for every *B. thuringiensis* genomic DNA sample analyzed (Fig. 2).

DGGE Optimization

The optimal time of electrophoresis and the range of denaturant agent gradient were determined by parallel gradient electrophoresis. In this case, the denaturing gradient is parallel to the electric field and the range of denaturants was narrowed, allowing a better separation [14].

The optimal time of electrophoresis allowing a good separation of the fragments was experimentally determined by loading different samples in the gel at constant time intervals. In this work a parallel DGGE analysis of a mixture of three *B. thuringiensis* strains were carried out to determine an appropriate gradient of denaturant concentrations for OL3-I-CG/OL5 primer pair. We determined that the optimal denaturant gradient was 30–65% for the

primer pair OL3-I-CG/OL5. We performed time of travel experiments with the same mix of PCR products from this *B. thuringiensis* strains to determine the optimal electrophoresis time. After 10–12 h at 100 V good resolutions were obtained (Fig. 1a–c); however, a small improvement was observed after 18–22 h when bands were clearly defined (Fig. 3 and data not shown). Hence, the electrophoresis conditions used were 60 V for 22 h for that primer pair.

Analysis of PCR Products by DGGE

PCR-DGGE analyses of *cry*-related sequences of various *B. thuringiensis* strains showed that there were sufficient differences in the migration of the amplicons to discriminate among the majorities of the putative *cry* gene fragments present in each strain (Fig. 3). It also showed a reproducible singular band-profile for single *B. thuringiensis* strains.

Most of the bands detected after the PCR-DGGE analyses of the reference strains *B. thuringiensis* sp. *kurstaki* (HD1) and *tenebrionis* (Btt) and of the FCC 41 native strain were cut out of the gels, purified and cloned. The cloned fragments were sequenced, and they were identified as partial *cry* genes after BLAST N and multiple-sequence alignment analysis. In this way, analyzed HD1 sequences obtained were identical to the corresponding regions of *cry1Ia* and similar to *cry1D* and other *cry1A* genes; Btt sequences were identical to *cry3Aa* genes and the two partial sequences from native strain FCC41 were identical to *cry24Ca* gene previously described [http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt], when were analyzed by BLAST N. We obtained more than one band for each gene fragment, increasing this way of being able informative. Multiple bands were obtained for the same *cry* genes as part of the amplification approach as a result of the use of degenerate primers. Differences at the primer sequences (one to four) was enough to give rise to discrete bands after DGGE analysis, as confirmed by sequencing assays. Nevertheless, the profile obtained for each strain under the same DGGE conditions used in this work was unchanged, indicating the reproducibility of the technique. Moreover, we found that the increase in the complexity of the band patterns after the combination of PCR with degenerate primers and DGGE makes more robust the assignment of a band profile to a specific *B. thuringiensis* strain.

Thus, the DGGE assay developed here provides a rapid and reliable way to analyze the genetic diversity of *cry* genes present in a single strain of *B. thuringiensis* in axenic cultures, as well as in mixtures of samples that it could include environmental ones. Although not analyzed in this work, this method might be adapted for the direct assay of
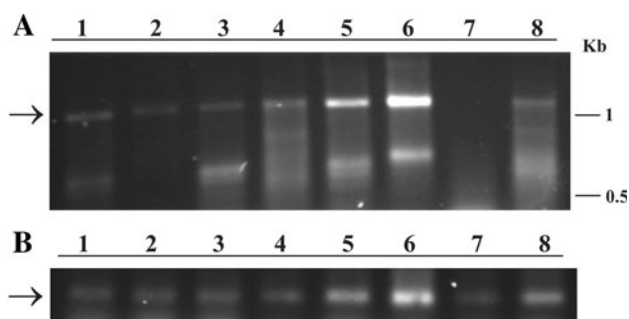

Fig. 2 PCR amplification products from different *B. thuringiensis* strains separated by agarose electrophoresis. **a** Amplified DNA fragments obtained with the primer pairs OL1-I/OL5 in a first PCR amplification step, using as template DNA from known *B. thuringiensis* strains. **b** PCR products obtained after amplification from DNA of known strains using primers OL-I 3 and OL5 in the second PCR amplification step. The positions of molecular weight markers (λ *Pst* ladder) are indicated. *Arrows* indicate the positions of the predicted amplified DNA fragments. *Lanes*: *1* FCC41, *2* HD1, *3* Btt, *4* H14, *5* 4E1, *6* 4J3, *7* 4F1 and *8* 4G1
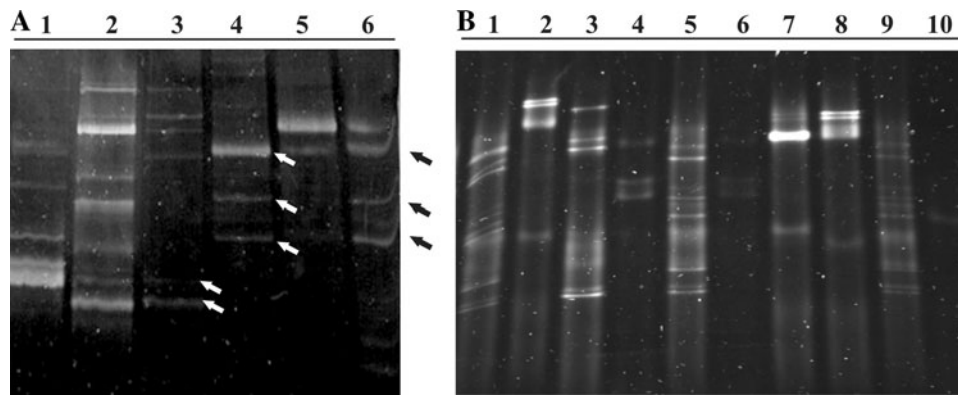
Fig. 3 DGGE fingerprint of *cry*-related sequences in a parallel gradient electrophoresis with denaturant gradient from 30 to 65% and electrophoresed at 60 V for 22 h. PCR products obtained after amplification from DNA of *B. thuringiensis* known strains by using primers OL-I 3 GC and OL5 in the second PCR amplification step. **a** *Lanes*: *1* FCC7, *2* FCC4, *3* FCC41, *4* Btt, *5* H14, and *6* HD1. **b** *Lanes*: *1* 4K1, *2* 4G1, *3* 4E1, *4* 4F1, *5* 4CF1, *6* 4T1, *7* 4W1, *8* 4J3, *9* 4AP1, and *10* 4M1. *Arrows* highlight excised bands in this work

environmental DNA for high throughput prospection of environmental samples containing potentially useful novel *cry*-genes. In addition, it may be useful as a monitoring tool for predicting the effects of agricultural practices on *B. thuringiensis* communities in soil.

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